

# Requirements for Efficient Correction of $\Delta$ F508 CFTR Revealed by Analyses of Evolved Sequences

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## SUMMARY

Misfolding of  $\Delta$ F508 cystic fibrosis (CF) transmembrane conductance regulator (CFTR) underlies pathology in most CF patients. F508 resides in the first nucleotide-binding domain (NBD1) of CFTR near a predicted interface with the fourth intracellular loop (ICL4). Efforts to identify small molecules that restore function by correcting the folding defect have revealed an apparent efficacy ceiling. To understand the mechanistic basis of this obstacle, positions statistically coupled to 508, in evolved sequences, were identified and assessed for their impact on both NBD1 and CFTR folding. The results indicate that both NBD1 folding and interaction with ICL4 are altered by the  $\Delta$ F508 mutation and that correction of either individual process is only partially effective. By contrast, combination of mutations that counteract both defects restores  $\Delta$ F508 maturation and function to wild-type levels. These results provide a mechanistic rationale for the limited efficacy of extant corrector compounds and suggest approaches for identifying compounds that correct both defective steps.

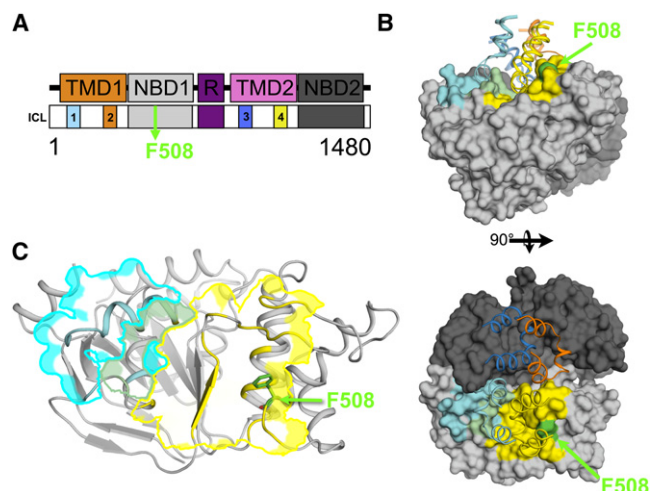
## INTRODUCTION

Cystic fibrosis (CF) is a lethal, monogenic disease caused by dysfunction of the CF transmembrane conductance regulator (CFTR). CFTR is a member of the ATP-binding cassette (ABC) transporter family that functions as a chloride channel. The protein is composed of five domains: two transmembrane domains (TMDs) that form the channel through the membrane, two nucleotide-binding domains (NBDs) that gate the channel through ATP-binding and hydrolysis reactions, and a regulatory region (R) that mediates kinase-dependent regulation of activity

(Figures 1A and 1B) (Berger et al., 2005; McCarty, 2000; Mendoza and Thomas, 2007; Riordan et al., 1989; Vergani et al., 2005). Most commonly, CF is associated with the deletion of a single amino acid, Phe508, in the first NBD (NBD1) of CFTR (Figures 1A–1C) (Riordan et al., 1989).  $\Delta$ F508 CFTR fails to fold properly and does not efficiently traffic to the plasma membrane (Cheng et al., 1990) resulting in a loss-of-function (Rich et al., 1990). The mutant CFTR is active when induced to fold and traffic to the membrane (Denning et al., 1992; Teem et al., 1993), indicating that compounds that correct the folding defect may have therapeutic benefit.

Studies of the NBD1 of CFTR indicate that the deletion reduces folding efficiency (Qu and Thomas, 1996; Thibodeau et al., 2005) and stability (Hoelen et al., 2010; Protasevich et al., 2010; Thibodeau et al., 2010; Thomas et al., 1992; Wang et al., 2010). Crystal structures of the isolated CFTR-NBD1 domains reveal that F508 is partially exposed on the surface (Lewis et al., 2004) and that deletion of the residue causes only local rearrangements in the loop following the 508 residue in the context of stabilizing mutations that facilitated crystallization (Atwell et al., 2010; Lewis et al., 2005).

Evidence suggests that  $\Delta$ F508 CFTR can be targeted to improve folding and restore function. A patient with a less severe form of CF had  $\Delta$ F508-R553Q on one allele and a nonsense mutation on the other (Dörk et al., 1991). A mating screen in yeast utilizing a CFTR-Ste6 chimera identified the same R553Q mutation as a suppressor of the loss-of-mating  $\Delta$ F508 phenotype (Teem et al., 1993). Additional second-site revertant mutations I539T, G550E, R553M, and R555K, within the portion of CFTR NBD1 included in the chimera, were also identified (DeCarvalho et al., 2002; Teem et al., 1993, 1996). The R553M, I539T, and the combination of G550E-R553M-R555K (3M) mutations correct the folding and stability defects of the  $\Delta$ F508 NBD1 domain in isolation (DeCarvalho et al., 2002; Hoelen et al., 2010; Pissarra et al., 2008; Qu et al., 1997; Thibodeau et al., 2010) but only partially restore maturation of the full-length mutant protein (Hoelen et al., 2010; Pissarra et al., 2008; Thibodeau et al., 2010). These data indicate that correction of the NBD1 folding



**Figure 1. Relationship of CFTR Domains to the F508 Position**

(A) CFTR is 1,480 amino acids in length and contains two transmembrane domains (TMDs, orange and pink) and four intracellular loops (labeled ICL1–4, cyan, orange, blue, and yellow), two nucleotide-binding domains (NBDs, gray and charcoal), and a highly disordered Regulatory region (R, purple). F508, green is located within the NBD1 domain.

(B) The Sav1866 based homology model of CFTR sans R region (PDBs 2HYD, 1R0W, and 3GD7). Top: view of the NBDs and ICLs of CFTR parallel to the membrane. The two NBDs associate in a head-to-tail fashion. ICL1 (cyan ribbon) and ICL4 (yellow ribbon) interact with NBD1 and ICL2 (orange ribbon) and ICL3 (blue ribbon) interact with NBD2. F508 (green surface) is at the predicted interface with ICL4. NBD1 surface residues predicted to be within 6 Å of ICL1, ICL4 or both are highlighted in cyan, yellow, or light green respectively. Bottom: view of the CFTR model looking down from the membrane (90° relative to top). See also Figure S1.

(C) Ribbon representation of NBD1 (PDB 1R0W) showing relationship of F508 (green sticks) to the predicted interface with ICL4 yellow outline.

defect alone only incompletely restores maturation of  $\Delta$ F508 CFTR.

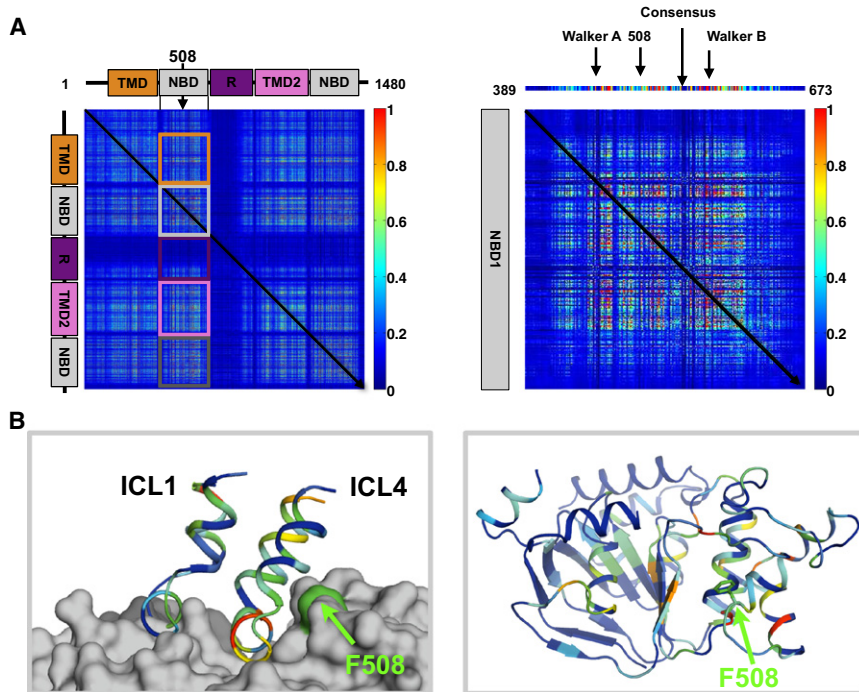
Based on the hypothesis that compounds that effectively improve the folding of  $\Delta$ F508 CFTR would be of therapeutic benefit, several cell-based high-throughput screens have been performed (Pedemonte et al., 2005; Robert et al., 2008, 2010; Van Goor et al., 2006). Although some of the compounds identified bind to and stabilize NBD1 (Sampson et al., 2011), the mechanism of action of most remains unknown. None of the reported  $\Delta$ F508 correctors have potency much less than 1  $\mu$ M and, like the single intragenic second site-suppressor mutants, efficacy above  $\sim$ 15% of wild-type levels (Van Goor et al., 2011). There is reasonable concern that this degree of correction will not provide therapeutic benefit based on the promising results with the more potent and efficacious potentiator of the rarer CF-causing G551D gating mutant (Accurso et al., 2010; Van Goor et al., 2009). This potentiator compound, VX-770, demonstrates that pharmacological improvement of mutant CFTR function in adult patients provides a measurable clinical benefit (Accurso et al., 2010), further stimulating the search for effective correctors of  $\Delta$ F508 folding and function. Understanding the molecular basis of the apparent correction ceiling and the mechanism of action of corrector compounds would provide critical insight for development of corrector compounds.

The identification of positions coupled to F508, provided by the Ste6-CFTR chimera revertant mating screen, has provided insights into CFTR folding and the effect of  $\Delta$ F508 but was limited to residues immediately proximal to the F508 position (Teem et al., 1993, 1996). Extending such a second-site analysis to the whole of CFTR might be expected to provide new information about the folding and maturation of CFTR and the effect of the  $\Delta$ F508 mutation thereon—information critical for revealing the mechanism of action of extant correctors, for developing methods capable of identifying better compounds, and for a fundamental understanding of the process of membrane protein folding. In the absence of a conveniently assessed phenotype for CFTR function as required to evaluate millions of individual genetic perturbations, the information nature has provided in the sequences of the ABC supergene family offer an alternative. Multiple algorithms have been developed that can identify residues coupled to specific positions, for example F508, in the evolved sequences of protein homologs (Fodor and Aldrich, 2004; Goh et al., 2000; Kass and Horovitz, 2002; Lockless and Ranganathan, 1999; Pazos and Valencia, 2008; Socolich et al., 2005). The set of residues statistically coupled to the F508 position would be expected to be enriched for positions that interact directly or allosterically in the final structures or transiently during the process of folding. Such interactions should be evident in the stability and folding yield of NBD1 and the maturation of CFTR, which reflects the association of folded NBD1 with ICL4. In the present work we identified positions coupled to F508 in CFTR and determined their effect on both NBD1 folding and CFTR maturation. The results reveal the quantitative effect of the  $\Delta$ F508 mutation on two distinct steps of folding and, further, suggest mechanistically defined methodologies for identifying compounds targeting either or both steps defective in the mutant protein.

## RESULTS

### Positions Coupled to 508 in the ABC Transporter Supergene Family

CFTR is a member of the ABC transporter supergene family (Dean and Annilo, 2005). Over 2,000 full-length sequences of eukaryotic ABC transporters (containing two NBDs and two TMDs on a single polypeptide) were obtained from iterations of a PSI-Blast default search using the human CFTR protein sequence as the seed (gi 90421313). Four multiple sequence alignments (MSA) were generated using ClustalW (Larkin et al., 2007). After quality assessment using the three highly conserved Walker A and B, and consensus motifs (Dean and Allikmets, 1995; Dean and Annilo, 2005; Jones and George, 2004; Walker et al., 1982), the sequences to be queried was reduced by half. Removal of additional sequences that had  $>90\%$  identity to any other sequence, produced a set of 493 full-length sequences (MSA available for download at <http://cftrfolding.org>). The NBD domains have the highest number of conserved positions in the MSA. Four positions in CFTR are invariant in the alignment, K464 and G1244 in the Walker A motifs of NBD1 and NBD2, respectively, Q1292, the Q loop residue in NBD2, and D1370 of the NBD2 Walker B motif. The TMDs have high conservation at or near the ICLs. The scores in the R domain approach



**Figure 2. CFTR Inter-Residue Coupling Matrix Calculated from an ABC Transporter Sequence Alignment**

(A) Left: heat map of the 1,480 × 1,480 matrix representing a linear combination of statistical methods (SCA, ELSC, OMES, McBASC). The color scale (right) with statistical scores approaching zero represented by dark blue and scores of high statistical significance in red. The diagonal denotes identity. Coupling of each of the five domains of CFTR to NBD1 are shown in squares with TMD1 in orange, NBD1 coupling to itself in light gray, the R region in purple, TMD2 in pink, and NBD2 in dark gray. See also Figure S2. Right: the 285 × 285 matrix of coupling scores within NBD1 (positions 389–673 of the left panel). Above the matrix is the column of coupling scores for every position in NBD1 to the 508 position. The conserved Walker A and B, consensus motifs, and the 508 position are denoted by arrows.

(B) Left: the 508-coupling scores in ICL1 (residues 158–184) and ICL4 (residues 1050–1080) of the CFTR model (Figure 1B). The surface corresponding to the F508 position is colored in green and is predicted to be in contact with ICL4. Right: view of NBD1 (PDB 1R0W) colored with normalized scores from (A). The F508 position is shown in green.

randomness (conservation score of  $-1$ ). Phe is the most common amino acid at the 508 position followed by Tyr at frequencies of 0.86 and 0.11, respectively.

Matrices of the pairwise correlation scores were calculated using the MSA by four independent, statistical methods, statistical coupling analysis (SCA), explicit likelihood of covariation (ELSC), OMES, and McBASC (Figures 2A and 2B; Figure S2 available online) (CSV files are available for download at <http://cftrfolding.org>) (Dekker et al., 2004; Fodor and Aldrich, 2004; Lockless and Ranganathan, 1999). The top 20 statistical 508-coupled positions for the four methods formed a set of 45 positions (Table 1). A random set of 16 of these 45 508-coupled positions (Table 1; Figure S3), were assessed for effects on CFTR folding. The 16 positions represent  $\sim 78\%$  coverage of the top ten 508-coupled positions for all four methods and include six positions common between all four methods, two positions between three methods, six positions between two methods, and two positions unique to a single method (Table 1). The six coupled positions common between all four methods are located in the  $\alpha$ -helical subdomain (Table 1; Figure S3). Ten of the sixteen positions are surface exposed; F490L and W496, like F508, are predicted to be at the ICL4 interface (Table 1; Figure S3). Additional positions reside within the coupling helix boundaries of ICL1 and ICL4 (Figure 2B). Each of the 16 positions in CFTR was mutated to the most common non-CFTR amino acid (Table 1), and the effect on both NBD1 folding and CFTR maturation was assessed.

#### Mutations at 508-Coupled Positions Affect NBD1 Folding

The 16 508-coupled positions and previously identified second-site suppressor mutations, and combinations thereof, were

evaluated for effects on the folding of the isolated human CFTR NBD1 using a structural complementation assay previously described (Thibodeau et al., 2010; Wigley et al., 2001). In the current implementation of the assay, the folding signal of  $\Delta F508$  NBD1 was  $0.43 \pm 0.03$  relative to wild-type NBD1,  $1.00 \pm 0.03$ . All 16 508-coupled and second-site suppressor mutations had a significant effect on the folding of the isolated domain relative to wild-type (Figure 3A), suggesting that the statistical analyses identified a set enriched for biological relevance. Fourteen of the 508-coupled mutations interfered with NBD1 folding (Figure 3A, green bars). The significance of the decrease in folding was  $<6E^{-6}$  for 13 of the mutants and 0.002 for the least significant, E583G. Two 508-coupled mutants, D529F and S573E, dramatically increased the relative folding yield of NBD1 by  $3.07 \pm 0.13$ -fold and  $1.85 \pm 0.06$ -fold, respectively, a level comparable to that of the second-site suppressors identified in the STE6 chimera screen (Figure 3A).

Thermal denaturation of purified D529F and S573E NBD1 revealed that S573E raised the  $T_m$  by  $2^\circ\text{C}$ – $5^\circ\text{C}$  relative to wild-type depending on ATP concentration (Figure S4B), indicating the improved folding yield in the complementation assay could be accounted for by increased stability of the native state (Figure 3B). In stark contrast, D529F had no measurable effect on  $T_m$  indicating the complementation assay reflects changes in yield and pathway as well as changes in stability or solubility. D529 is located in the  $\alpha$ -helical subdomain of NBD1 near the NBD1-NBD2 interface (Figure 1C). Analysis of the human NBD1 crystal structures (PDB 2BBO and 1XMI) reveals this aspartic acid side chain forms a salt bridge with R555. The R555 side chain also makes a hydrogen bond with the backbone carbonyl of Q525 (Figure S4A). The R555K missense mutation



**Table 1. Top 20 508-Coupled Positions within NBD1 Using Four Independent Statistical Methods**

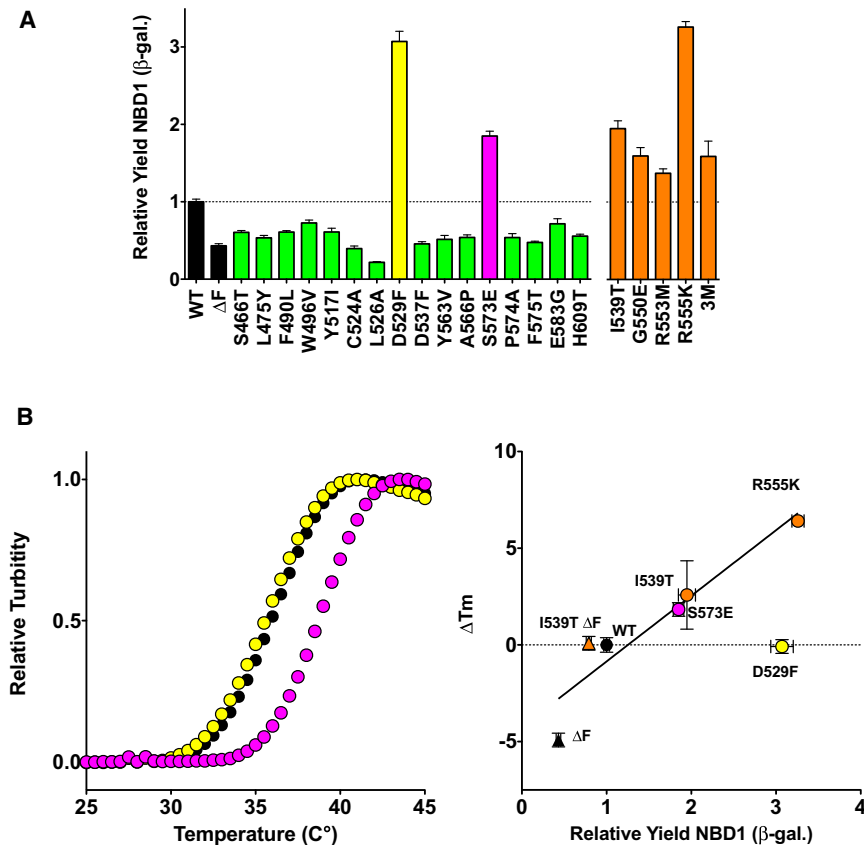
ELSC	McBASC	OMES	SCA
			435
		453	
		460	
		<b>S466T</b>	<b>S466T</b>
	468		
		470	
	472		
		<b>473</b>	473
		474	474
<b>L475Y</b>		<b>L475Y</b>	<b>L475Y</b>
	<b>F490L</b>		<b>F490L</b>
<b>W496V</b>	<b>W496V</b>	<b>W496V</b>	<b>W496V</b>
	503		
	505		
	<b>507</b>		
	<b>509</b>		
512			
513			
<b>Y517I</b>	<b>Y517I</b>	<b>Y517I</b>	<b>Y517I</b>
		520	520
	<b>521</b>		
<b>C524A</b>	<b>C524A</b>	<b>C524A</b>	<b>C524A</b>
<b>L526A</b>	<b>L526A</b>	<b>L526A</b>	<b>L526A</b>
<b>D529F</b>	<b>D529F</b>	<b>D529F</b>	<b>D529F</b>
<b>D537F</b>			<b>D537F</b>
	<b>543</b>		
<b>Y563V</b>	<b>Y563V</b>	<b>Y563V</b>	<b>Y563V</b>
<b>A566P</b>		<b>A566P</b>	
		<b>569</b>	569
			<b>S573E</b>
<b>P574A</b>		<b>P574A</b>	<b>P574A</b>
<b>F575T</b>		<b>F575T</b>	
			578
582			
<b>E583G</b>		<b>E583G</b>	
587			
	591		
	595		
	598		
	602		
604		<b>604</b>	604
<b>H609T</b>			
	617		
630			630
640			

The alignment of 493 sequences was used to calculate pairwise coupling scores using each method (ELSC, SCA, McBASC, and OMES). The top 20 are shown for each method, the top 10 positions in bold. The tested set of 16 positions are underlined. See also Figure S3.

is one of the previously studied second-site suppressors of  $\Delta F508$  NBD1 mediated misfolding (Roxo-Rosa et al., 2006; Teem et al., 1996). R555K increased the folding yield of NBD1,  $3.26 \pm 0.07$ -fold over wild-type (Figure 3A). The  $T_m$  of R555K NBD1 was  $6.4^\circ\text{C}$  higher than wild-type in 2 mM ATP. A Glu or Asp at the CFTR equivalent 573 position is the catalytic base in the highly conserved Walker B motif in other ABC transporters (Jones and George, 2004; Mendoza and Thomas, 2007; Walker et al., 1982). The serine present in CFTR at this position accounts for the lack of ATP hydrolysis at this site as central to the mechanochemistry of the gating cycle (Figure S4B) (Vergani et al., 2003).

### Mutations at 508-Coupled Positions Affect Maturation

To examine the relationship of NBD1 folding and stability with full-length CFTR folding, mutant CFTRs were expressed in HeLa cells, and CFTR maturation was determined by two methods. As CFTR folds, it traffics from the ER to the Golgi where it attains complex glycosylation prior to reaching the plasma membrane. The resulting increase in molecular mass can be detected by western blotting of SDS-PAGE (Figure 4). The slower migrating complex glycosylated Band C reflects the folded, mature CFTR whereas the more rapidly migrating Band B reflects core glycosylated CFTR that has not reached the Golgi (Cheng et al., 1990). These data have been utilized in several ways to monitor the effect of mutations and compounds on CFTR folding. The ratio of Band C to Band B is linear with both CFTR at the surface as monitored by biotinylation and with Band C itself (Figure S5C). By contrast the ratio of Band C over total CFTR has a nonlinear relationship with mature surface CFTR, being more sensitive to changes at low folding yields (Figure S5B). In these analyses it was noted that none of the mutants tested had a significant effect on the amount of Band B and thus, the total CFTR was linear with the amount at the surface (Figure S5C). An ELISA assay for total CFTR quantitation was developed and used to monitor CFTR maturation. The signal from this method is not only linear but has lower noise than other methods (Figure S5C). The influence of all the 508-coupled mutants on CFTR folding was assessed (Figure 4A). All 16 of the 508-coupled mutations and second-site suppressor mutations altered the yield of full-length CFTR relative to wild-type. The same 14 mutations that reduced the yield of NBD1 folding, reduced maturation of full-length CFTR (Figure 4A, green bars). The p value for all is  $<0.04$ . The same two mutations that improved NBD1 folding yield, D529F and S573E, increased the maturation efficiency of CFTR (Figure 4A, yellow and magenta bars, respectively). The previously identified second-site suppressor mutants also improved CFTR maturation in a manner consistent with their effects on NBD1 folding. The correlation between NBD1 folding and CFTR maturation is formalized in Figure 4B. The data indicate a linear relationship ( $R = 0.85$ ) with a slope of 0.75. Thus, NBD1 folding is necessary for CFTR folding and for every fold change in NBD1 yield a concomitant change of 0.75 in CFTR yield is observed. The position along the x axis thus reflects the amount of folded NBD1 and the slope of the line likely reflects later steps in folding during which folded NBD1 associates with other parts of CFTR.



**Figure 3. Effects of 508-Coupled Mutations and Second-Site Suppressors on NBD1 Folding**

(A) The relative yield of NBD1 folding was determined using a cell-based assay. All mutations altered the relative yield relative to wild-type NBD1 as shown in the bar chart ( $\pm$  standard error of the mean [SEM],  $n = 9$  except for WT,  $\Delta F508$ , D529F, and S573E where  $n = 18$ ). Two of the 508-coupled positions, D529F and S573E, increase the yield of NBD1. The remaining mutations decrease the yield of soluble NBD1.

(B) Thermal denaturation of 5  $\mu$ M purified recombinant WT, D529F, and S573E NBD1 in the presence of 2 mM ATP (left panel). See also Figure S4. By contrast to the increased NBD1 folding yield, the D529F mutation (yellow circles) has no observable effect on thermal stability relative to WT (black circles). S573E (magenta circles) increased the melting temperature 2°C. Correlation between thermal stability of bacterially expressed and purified NBD1 and NBD1 folding yield (right panel). For mutants on either WT (circles) or  $\Delta F$  (triangles) backgrounds, the correlation between stability and folding yield,  $R = 0.94$  when D529F is excluded (yellow circle) (right panel,  $\pm$  SEM).

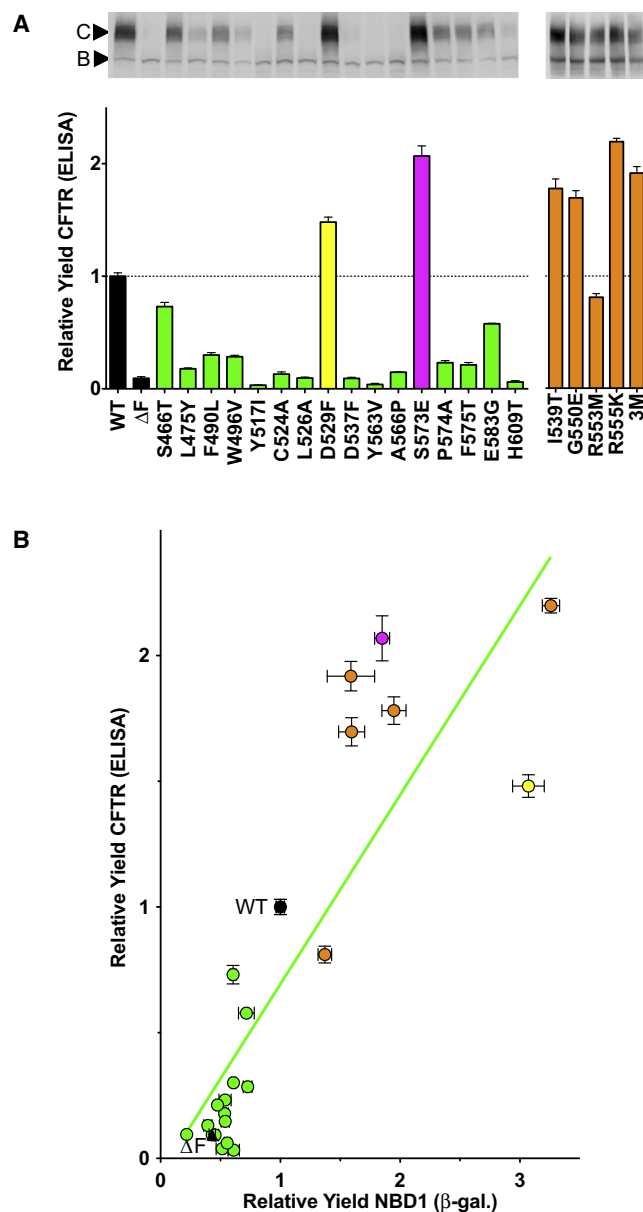
### Influence of $\Delta F508$ on NBD1 Folding and Later Steps in CFTR Maturation

To determine whether in addition to its known effects on NBD1 folding and stability,  $\Delta F508$  interferes with later steps in CFTR folding, the differential effects of the NBD1 suppressor mutations on the wild-type and  $\Delta F508$  NBD1 folding yield and CFTR maturation yield were measured and correlated (Figure 5B). Interestingly, the two suppressors identified by the coupling analysis, D529F and S573E, had much more modest effects on NBD1 yield in the presence of the  $\Delta F508$  mutant as might be expected. Taken together, the suppressors define a line ( $m = 0.10$ ,  $R = 0.36$ ) in the context of  $\Delta F508$ , indicating that the mutation interferes with a later step in CFTR folding in addition to its defect in NBD1 folding yield. Thus, the mutation decreases NBD1 folding yield  $\sim 3$ -fold and later CFTR folding steps by  $\sim 7$ -fold.

Extant structures of CFTR  $\Delta F508$  NBD1 contain a series of previously identified second-site mutations that improve folding and solubility of the model domain. To simplify structural interpretation of the current results, a high resolution structure of murine  $\Delta F508$  NBD1 without additional mutations or deletions was solved (Table S1). Comparison of the backbones of the murine wild-type (PDB 1R0W) and  $\Delta F508$  NBD1 (PDB 3S17) structures reveal nearly identical structures, root-mean-square (rms) 0.249 Å, with backbone shifts immediately following the 508 position. The largest shift in  $C_{\alpha}$  between the two structures is G509 shifting by 3.5 Å followed by a 1.5 Å shift for V510 before the  $\Delta F508$  backbone converges with wild-type. The surface view

of the two structures suggests local changes near the site of the deletion (Figure S1) similar to the structures containing the second-site mutations (Lewis et al., 2010, 2005). The absence of the F508 side chain creates a significant pocket on the surface of NBD1 at the predicted interface between ICL4 and NBD1 (Figure S1B). Additionally, the V510 side chain rotates outwardly 3.6 Å ( $C_{\beta}$ ) from the core in the mutant structure, further increasing the size of the pocket.

To better understand the molecular basis of the later step in folding effected by the  $\Delta F508$  mutation, we built upon earlier work indicating that most missense mutations at the 508 position do not have dramatic effects on NBD1 but rather interfere with CFTR maturation (Du et al., 2005; Thibodeau et al., 2005). The current assay battery was applied to a set of these mutations alone or in combination with the NBD1 suppressors (Figure 5C). When F508 is replaced by Lys, very little CFTR maturation is observed regardless of the ability of the NBD1 to fold ( $m = 0.03$ ,  $R = 0.38$ ). The model of CFTR (Mendoza and Thomas, 2007) based on alignment with Sav1866 (Dawson and Locher, 2007) places F508 (or the  $\Delta F508$  pocket) near R1070 in ICL4 (I218 in ICL2 of Sav1866) (Figure 5C, top). Residues in ICL4 coupled to the 508 position exhibit a helical pattern consistent with the structural model. To assess the NBD1-ICL4 interface from the ICL4 side of the interface, a large tryptophan side chain replaced the native arginine. The R1070W sterically clashes with the F508 position in the model (Figure 6A, top) a prediction consistent with fact that it is a CF-causing mutation (Krasnov et al., 2008) that inhibits CFTR maturation when F508 is present (open circle, Figure 6A, bottom). By contrast, the introduction of the larger side chain at 1070 is accommodated by the  $\Delta F508$



**Figure 4. Effects of 508-Coupled and Second-Site Suppressor Mutations on CFTR Maturation: Correlation with NBD1 Folding Yield**

Effects of 508-coupled mutations (green, yellow, and magenta) and second-site suppressors (orange) on the maturation of full-length CFTR and correlation with NBD1 folding yield.

(A) The efficiency of full-length CFTR maturation was determined by ELISA ( $\pm$ SEM,  $n = 6$  for WT,  $\Delta$ F508, D529F, and S573E,  $n = 3$  for other mutants). A control western blot is shown above the bar graph. All mutations altered maturation relative to wild-type CFTR. The two 508-coupled positions that increased NBD1 folding yield, D529F and S573E, also increased the maturation yield of CFTR (yellow and magenta bars, respectively). See also Figure S5. (B) The influence of the 508-coupled mutations (green circles), four second-site suppressor mutations (I539T, G550E, R553M, and R555K) and three suppressors in combination (G550E, R553M, and R555K) (orange circles) on F508 background on relative maturation of full-length CFTR and relative NBD1 folding yield is correlated (green line,  $m = 0.75$ ,  $R = 0.85$ ). The two F508-coupled position mutations, D529F and S573E, are colored in yellow and magenta circles, respectively.

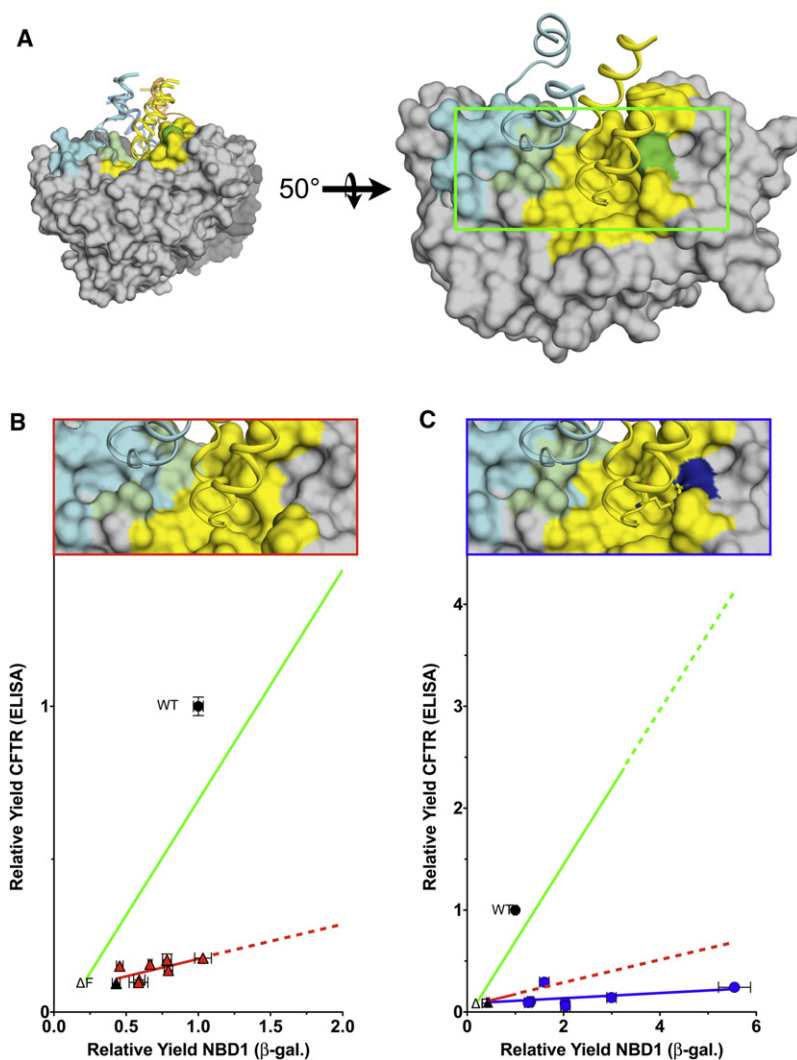
pocket in the mutant NBD1, and, could allow for better packing (Figure 6B, top) that promotes folding (open triangle, Figure 6A, bottom) by correcting the ICL4- $\Delta$ F508-NBD1 interface defect.

The ability of the R1070W mutation to counteract the ICL4-NBD1 interface defect caused by the  $\Delta$ F508 mutation, allowed assessment of the quantitative effects of suppression of both defects concurrently. When NBD1 suppressor mutations were introduced on top of R1070W and  $\Delta$ F508, the slope was restored ( $m = 0.77$ ,  $R = 0.47$ ) (Figure 6B). Thus, correction of the two defects results in near total rescue of the mutant folding phenotype. A 3-fold improvement in  $\Delta$ F508 NBD1 folding yield, correcting the NBD1 to wild-type levels, results in a modest improvement in CFTR maturation (0.10 slope). Similarly, the R1070W interface mutant produces an  $\sim 7$ -fold increase in the slope, but only a modest improvement in CFTR maturation. However, when the two effects are combined, they have a multiplicative effect on folding.

CF is a loss-of-function disease. To determine whether efficient correction of maturation was adequate to restore function, CFTR activity was measured for mutations that corrected either the NBD1 defect or the ICL4-NBD1 defect or both. Two assays were employed: transepithelial ion conductance was determined in FRT cell monolayers (Figure 6C) and whole-cell currents were measured in HeLa cells (Figure 6D). CFTR dependent function was taken as the forskolin dependent, Inh-172 sensitive activity. Mirroring the maturation results, correction of either the NBD1 defect (I539T, R555K, red bars) or the ICL4-NBD1 defect (R1070W, white bar) alone provided only modest improvements of function. Like maturation, combination of the two types of correction mutations (cyan bars) had a synergistic effect, restoring CFTR function to near wild-type levels.

## DISCUSSION

The most common CF-causing mutation,  $\Delta$ F508, disrupts the normal folding pathway of CFTR leading to the loss-of-function that underlies pathogenesis (Cheng et al., 1990). The mutant protein is active when induced to fold (Denning et al., 1992; Teem et al., 1993) suggesting a novel pharmacological approach to treating CF. Small molecules that bind to and counteract the folding  $\Delta$ F508 defect would provide a means of repairing the protein (Thomas et al., 1992) while maintaining normal regulation and expression patterns. Multiple efforts to identify such compounds have been reported (Pedemonte et al., 2005; Robert et al., 2010, 2008; Sampson et al., 2011; Van Goor et al., 2006), but the identified molecules are not very potent and correct mutant CFTR folding to no more than 15% of wild-type (Van Goor et al., 2011). Understanding the basis of this observed efficacy ceiling would provide information critical for determining the feasibility of the corrector approach and guide any future discovery efforts. Although it is known that CFTR folding is a hierarchical process that occurs partially during translation (Du et al., 2005; Hoelen et al., 2010; Thibodeau et al., 2005) and that the  $\Delta$ F508 mutation interferes with NBD1 folding (Hoelen et al., 2010; Protasevich et al., 2010; Thibodeau et al., 2005; Thomas et al., 1992) and is reflected in other domains (Du et al., 2005), fundamental details of the folding pathway and its disruption are lacking.



**Figure 5.  $\Delta$ F508 and 508 Missense Mutations Influence the Correlation of CFTR Maturation with NBD1 Folding Yield**

(A) View of the CFTR NBD1-ICL1/4 interface, as described in Figure 1B.

(B) Deletion of the F508 residue creates a pocket on the NBD1 surface near the predicted ICL4 interface (compare  $\Delta$ F508 model built with PDB 3SI7, to wild-type Figure 1C, PDB 1R0W). See also Table S1. Previously identified second-site suppressor (I539T, G550E, R553M, R555K, and 3M) but not the 508-coupled mutants (D529F and S573E) increase the yield of  $\Delta$ F508 NBD1. The correlation between increased NBD1 yield and relative yield of  $\Delta$ F508 CFTR is shallower (red line,  $m = 0.10$ ,  $R = 0.36$ ) than wild-type (green line,  $m = 0.75$ ,  $R = 0.85$ ) ( $\pm$ SEM). See also Table S2.

(C) F508K, F508R, and F508K in combination with I539T, G550E, R553M, R555K, and 3M mutations increase folding yield of NBD1, but exhibit no corresponding increase in CFTR maturation yield (dark blue circles and line,  $m = 0.03$ ,  $R = 0.40$ ) ( $\pm$ SEM,  $n = 9$  along x axis and  $n = 3$  along y axis).

To gain insight, we identified residues coupled to the 508 position utilizing a computational approach. Several methods have been developed to use evolutionary sequence analysis to identify networks of residues within proteins important for function, to improve native intermolecular contact prediction algorithms, and to identify hot spots of ligand binding or protein-protein interactions (Dekker et al., 2004; Goh et al., 2000; Lockless and Ranganathan, 1999; Madaoui and Guerois, 2008; Pazos and Valencia, 2008; Socolich et al., 2005). We used the ABC transporter family to identify positions related to F508 and its important role in folding. Multiple evolutionary coupling algorithms were utilized to identify relevant positions. Remarkably, the 16 508-coupled mutants selected and tested all effect the folding of both the isolated domain and the full-length CFTR. Fourteen mutations made folding worse and two improved the folding. These results are not likely to occur by random chance. For example, two random mutant screens found 0.03% of clones in the library improved folding, whereas 20% of mutants misfolded with an average of two mutations per clone, demon-

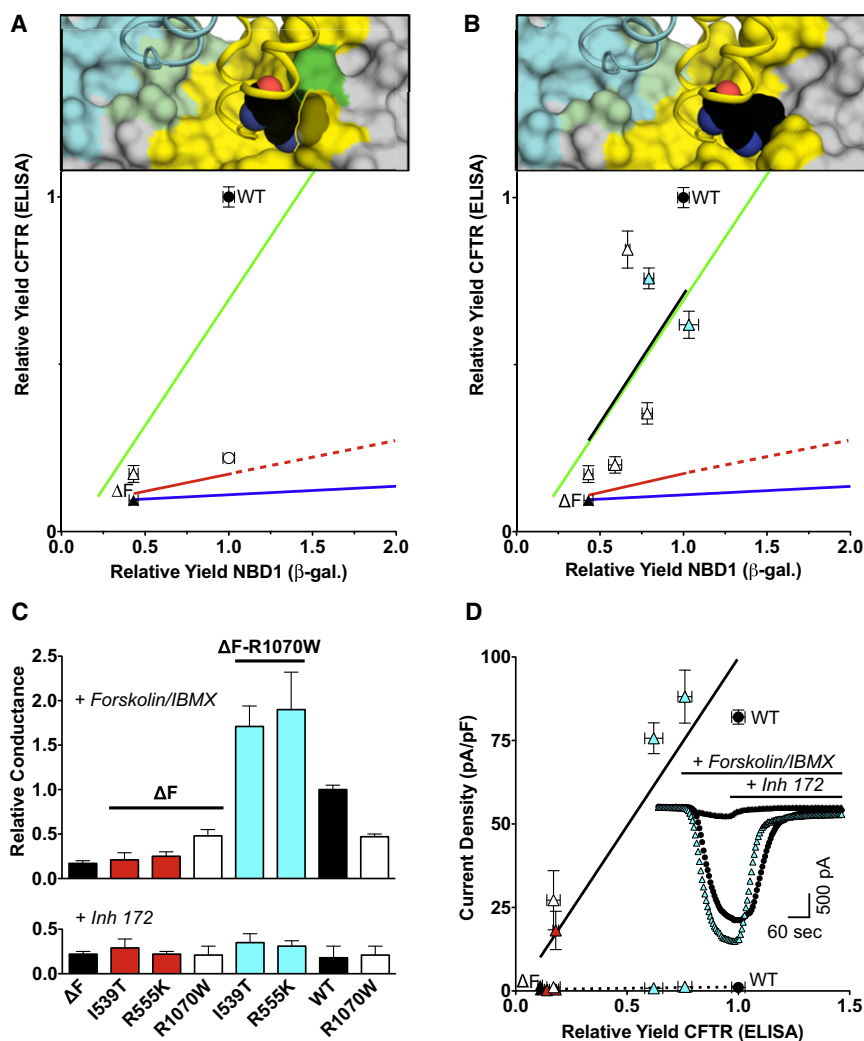
strating the inherent mutational robustness of proteins (Kunichika et al., 2002; Pjura et al., 1993) and the utility of the computational methods. In comparison to the random screens, we were 400 times more likely to identify mutations that improve folding and four times more likely to identify mutations that result in misfolding.

Identified in this study, D529F and S573E improve folding of NBD1 in isolation and maturation of full-length CFTR in the wild-type background. The human CFTR 573 position corresponds to a highly conserved Glu, the catalytic base, of the Walker B motif in other members of the ABC transporter family and in the second NBD of CFTR (Walker et al., 1982). However, CFTR proteins have a serine at this

position, accounting for the lack of ATPase activity at this site. Reversion to Glu does not restore catalytic activity at this site indicating there are other changes important in the evolution of CFTR as a channel from a family of transporters (Stratford et al., 2007; Vergani et al., 2003). In this work, we found the wild-type protein is less stable than the mutant containing the conserved residue (Figure 3B, leftmost panel; Figure S4B).

Analyses of identified coupled positions in the wild-type and  $\Delta$ F508 backgrounds, utilizing methodologies to facilitate the large number of samples, demonstrated that there are two steps in CFTR folding in which  $\Delta$ F508 is defective, NBD1 folding and a later step involving NBD1 and ICL4. The correction of only one defect by suppressor mutations or compounds has limited efficacy as is reflected in the partial maturation and function of  $\Delta$ F508 CFTR to <20% of wild-type, the previously observed "ceiling." In plots of CFTR folding yield against NBD1 folding yield, the position along the x axis reflects the first step in folding and the slope of the line reflects the later steps including the NBD1-ICL4 interface.





**Figure 6. Correction of the  $\Delta$ F508 NBD1-ICL4 Interface Defect Is Synergistic with Correction of the  $\Delta$ F508 NBD1 Folding Yield Defect**

(A) CFTR maturation yield as a function of NBD1 folding yield for wild-type,  $\Delta$ F508, and the ICL4-NBD1 interface mutants are shown as green, red, and dark blue lines for comparison. The R1070W (open triangle) mutation in ICL4 modestly improves the relative yield of  $\Delta$ F508 CFTR maturation and decreases the relative yield for CFTR wild-type (open circle) consistent with the predicted steric clash with the F508 side chain (top) ( $\pm$ SEM). See also Table S2.

(B) Top: by contrast, in the model Trp is accommodated by the pocket formed by the  $\Delta$ F508 mutant (PDB 3SI7) See also Table S1. When R1070W is combined with mutations that improve  $\Delta$ F508 NBD1 folding yield, I539T, G550E, R553M, R555K, and 3M (open triangles), the correlation between NBD1 folding and CFTR maturation in the wild-type protein is restored ( $m = 0.77$ ,  $R = 0.47$ , black line) ( $\pm$ SEM). See also Table S2.

(C) CFTR-dependent transepithelial conductance. Monolayers of FRT cells transiently expressing CFTR were stimulated with 10  $\mu$ M Forskolin + 100  $\mu$ M IBMX and transepithelial conductance measured (top,  $\pm$ SEM,  $n = 3$ , average conductance for wild-type = 1.24 mS). The CFTR dependence of conductance is indicated by the sensitivity to 20  $\mu$ M Inh-172 (bottom). See also Table S2.

(D) Forskolin stimulated, Inh-172 sensitive whole-cell currents of HeLa cells transiently expressing CFTR reveal a correlation between current density after activation (10  $\mu$ M forskolin + 100  $\mu$ M IBMX) with relative yield of CFTR (black line,  $\pm$ SEM,  $n = 5-6$ ) and in the presence of Inh-172 (10  $\mu$ M) dotted line ( $\pm$ SEM,  $n = 4-6$ ). A representative trace of the corrected mutant,  $\Delta$ F508-I539T-R1070W CFTR (cyan triangles) is more like wild-type (filled circles) than  $\Delta$ F508 (filled triangles) (inset). See also Table S2.

The experiments with the second-site suppressor mutations on the F508K background demonstrate, regardless of how well NBD1 folds, CFTR is unable to mature if the NBD1-ICL4 is disrupted. Likewise, CFTR cannot mature if NBD1 is unable to form a native structure. These findings predict that screens for compounds that improve CFTR maturation or function might be biased against compounds that act specifically against either individual folding defect as they would produce improved position on the x axis or a steeper slope but little actual improvement of CFTR maturation and function. Unless single compounds can correct both steps, the set of highest hits in such screens are likely to be populated with compounds that do not directly act on CFTR. Thus, these findings have implications for the discovery and development of CF therapeutics. Compounds, or combinations of compounds, that correct both defective steps could provide both on target specificity and efficacy of action. The assay systems and second-site mutants developed may also find utility in establishing a mechanism of action of extant correctors and aid in the identification of new compounds.

## EXPERIMENTAL PROCEDURES

### Bioinformatics

The human CFTR sequence (gi|90421313) was used as a seed for PSI-Blast (Altschul et al., 1997) of the nonredundant database. Subalignments were performed using ClustalW and assessed (Larkin et al., 2007). Sequences lacking conserved ABC transporter motifs (Walker A, B, and/or consensus motifs) (Walker et al., 1982) were removed. The final alignment was generated online using Promals 3D (Pei et al., 2008a, 2008b). Proteins >90% identity to any other protein in the alignment were removed. The alignment contains 493 sequences. Positional entropy based conservation scores were calculated using AL2CO (Pei and Grishin, 2001) and frequencies of the most common amino acid position identified and calculated via Perl scripting.

The multiple sequence alignment was used to calculate pairwise correlation matrices using SCA (Lockless and Ranganathan, 1999), ELSC (Dekker et al., 2004), OMES (Kass and Horovitz, 2002; Larson et al., 2000; Fodor and Aldrich, 2004), and McBASC (Göbel et al., 1994; Olmea et al., 1999). Pairwise correlation scores for SCA were calculated in MATLAB (The MathWorks) using the algorithm provided by Lockless and Ranganathan (1999). ELSC, OMES, and McBASC scores were calculated using the downloaded package from A. Fodor (<http://afodor.net>). Mutations at each of the positions were selected utilizing amino acid distribution profiles complementing correlated changes at the 508 position.



### NBD1 Folding Yield

pcDNA 3.1(+) constructs containing human WT and  $\Delta$ F508 CFTR NBD1 (residues 389–673) were used as previously described (Thibodeau et al., 2010; Wigley et al., 2001). HeLa TetOn (Clontech Laboratories) cells were transiently transfected using Lipofectamine 2000 (Invitrogen) at a cell density of 320,000 cells/ml. After transfection, cells were grown at 37°C for 18–24 hr. Media was aspirated from each well and 50  $\mu$ l of 2.5  $\mu$ M fluorescein-di- $\beta$ -D-galactopyranoside (FDG) (Sigma-Aldrich) substrate in Reporter lysis buffer (Promega) added. Fluorescence was measured every minute for 24 hr. Rates were determined from the linear portions of the data. p values were determined using the Student's t test with a two-tail distribution of a two-sample heteroscedastic test.

### CFTR Maturation Yield

With pCMV-CFTR (a gift from J. Rommens) HeLa TetOn (Clontech) cells were transiently transfected with Lipofectamine 2000 (Invitrogen) and grown at 37°C for 24 hr. Effects of mutations on maturation of CFTR was visualized by western blot analysis and quantitated by a sandwich ELISA utilizing mAb A596 (J. Riordan, UNC) as the capture antibody and 3G11 (W. Balch, Scripps) as the detection antibody. Characterization of the ELISA maturation assay and detailed methods are included in the [Extended Experimental Procedures](#) and [Figure S5](#).

### Protein Expression, Purification, and Characterization

Murine  $\Delta$ F508 NBD1, 389–673 human numbering, was expressed and purified as previously described except bacterial pellets were not frozen (Lewis et al., 2004). D529F, S573E, and R555K mutations of human wild-type NBD1 were expressed and purified as previously described (Thibodeau et al., 2005). Temperature melts were performed at 2 mM ATP (Hoelen et al., 2010).

### $\Delta$ F508 NBD1 Crystallization and Structure Determination

Crystals of murine CFTR NBD1  $\Delta$ F508 were grown using the vapor-diffusion method by mixing 1  $\mu$ l of 10 mg/ml of the murine  $\Delta$ F508 NBD1 protein with 1  $\mu$ l of the well solution (containing 3.5–4 M sodium acetate, pH 7.5) (Lewis et al., 2004; Thibodeau et al., 2005). Crystals used for data collection were flash-cooled by suspending in a nylon loop directly out of their mother liquor and then plunging into liquid propane. X-ray diffraction data were acquired at beamline 19-ID at the Structural Biology Center of the Advanced Photon Source at Argonne National Laboratories. Statistical details of the final model are found in [Table S1](#).

### Homology Model of CFTR

Murine NBD1 or  $\Delta$ F508 (PDB 1R0W or 3SI7) and human NBD2 (PDB 3GD7) were docked onto the Sav1866 structure (PDB 2HYD) (Dawson and Locher, 2007; Lewis et al., 2004). A set of sequences containing the ICL4 segments of CFTR proteins and Sav1866 ICL2 was used to generate an alignment using the JalView ClustalW function (Clamp et al., 2004; Waterhouse et al., 2009). On the WT or  $\Delta$ F508 CFTR models, F508K, R1070, R1070W residues were modeled using the PyMOL (Schrodinger, 2010) mutagenesis function (Figures 1B, 2B, rightmost panel, 5, 6, and S1). Contact distances between CFTR ICL1 and ICL4 residues and murine WT or  $\Delta$ F508 NBD1 residues were calculated via a Perl script using the atomic coordinates from the models.

### CFTR Function

The whole-cell patch-clamp technique was used to measure  $\text{Cl}^-$  current as previously described (Wang et al., 2006). Briefly, whole-cell conductance responses were measured in response to 10  $\mu$ M forskolin + 100  $\mu$ M IBMX and then 10  $\mu$ M Inhibitor-172 (Inh-172). Current recording and analysis was performed with pClamp 9.2 software and analyzed with Origin 8 software. Transepithelial conductance ( $G_t$ ) measurements across monolayers of transiently transfected FRT cells were performed using an EVOM<sup>2</sup> Epithelial Voltammeter with a STX100 electrode (World Precision Instruments) at 37°C.  $G_t$  was measured 10 min after treatment with 10  $\mu$ M Forskolin + 100  $\mu$ M IBMX and 5 min after 20  $\mu$ M Inh-172.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and two tables and can be found with this article online at [doi:10.1016/j.cell.2011.11.023](https://doi.org/10.1016/j.cell.2011.11.023).

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